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Original article

Synthesis and antibacterial activity of levofloxacin derivatives with certain bulky residues on piperazine ring

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Abstract

A number of levofloxacin analogues carrying a 2-aryl-2-oxoethyl or a 2-aryl-2-oxyiminoethyl moiety attached to the piperazine ring at C-10 position have been prepared and evaluated as antibacterial agents against a series of Gram-positive and Gram-negative bacteria. Some of them exhibited significant inhibitory activity against Gram-positive bacteria.

Keywords: Quinolones; Levofloxacin derivatives; In vitro antibacterial activity

1. Introduction

The increasing of resistance to many antibacterial agents seen currently is a great cause for concern. Gram-positive cocci have become a major problem, with resistance growing to β-lactams and to macrolides in strains of *Streptococcus pneumoniae* and staphylococci, especially in methicillin-resistant *Staphylococcus aureus* (MRSA) strains [1,2]. Thus, the design and development of new agents that provide effective therapy for infections caused by organisms resistant to older agents is an urgent need [3].

In the early 1960s, the discovery of nalidixic acid opened the door for a series of quinolone antibacterials. Nalidixic acid was first introduced quinolone for treatment of urinary tract infections caused by Gram-negative organisms [4]. The second generation of quinolones named fluoroquinolones, such as norfloxacin **1**, ciprofloxacin **2** and ofloxacin **3** (Fig. 1), resulted from fluorination at C-6 position and introduction of piperazine ring at C-7 position, have broad-spectrum activity for respiratory, urinary, gastrointestinal tracts, skin, and soft tissue infections caused by either Gram-negative or Gram-positive bacteria [5]. Most quinolones, such as norfloxacin **1**, and ciprofloxacin **2** possess a bicyclic heteroaromatic quinolone pharmacophore, but ofloxacin **3** is a certain tricyclic heteroaromatic compound with a third methyloxazine ring fused to quinolone and was initially marketed as the racemate [6]. However, the optically active *S*(−)-isomer, levofloxacin **4**, is 2-fold more potent than the racemate and 8−28-fold more potent than its *R*(+)-isomer [7,8]. The early fluoroquinolones, such as ciprofloxacin, have a broad spectrum of activity against Gram-negative bacilli and modest activity against Gram-positive cocci. The more recently released fluoroquinolone levofloxacin possess broad-spectrum antibacterial activity similar to that of earlier quinolones, however, it has enhanced activity against Gram-positive and atypical organisms [3,5].

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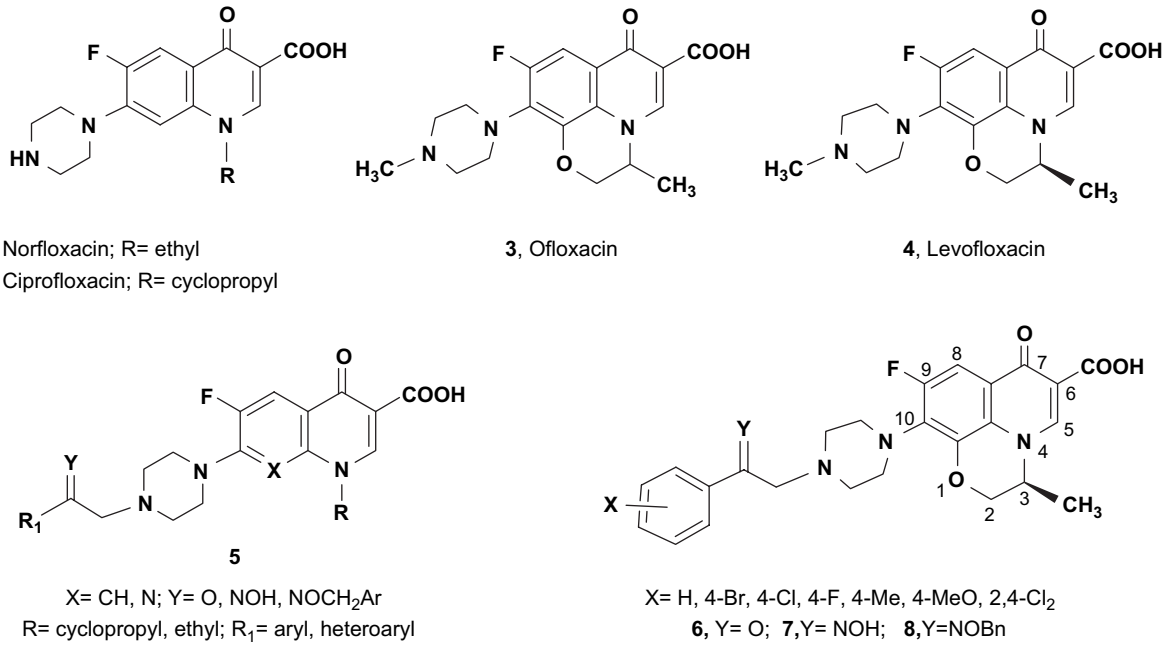


Fig. 1.

Recently, some strains that display resistance to fluoroquinolones have appeared. For example, failures have been reported when these fluoroquinolones have been used for the treatment of Gram-positive cocci, especially *S. pneumoniae* [9]. It is therefore important to search for more potent and broad-spectrum quinolone antibacterial agents capable of dealing with the resistant strains.

In the preceding papers [10–15], we described a number of *N*-substituted piperazinyl quinolones **5** by introducing a certain arylethyl moieties in the piperazine unit of 7-piperazinyl quinolones, ciprofloxacin, norfloxacin and enoxacin. Preliminary in vitro biological data indicated that some of these compounds exhibited high activity against staphylococci and in most of the cases, the activity of *N*-substituted piperazinyl quinolone depends on inherent activity of parent quinolone [10–15]. In the present study, we have aimed to achieve new quinolone antibacterials, by preparing the levofloxacin derivatives **6–8** carrying a 2-aryl-2-oxoethyl or a 2-aryl-2-oxyiminoethyl moiety attached to the piperazine ring at C-10 position (Fig. 1).

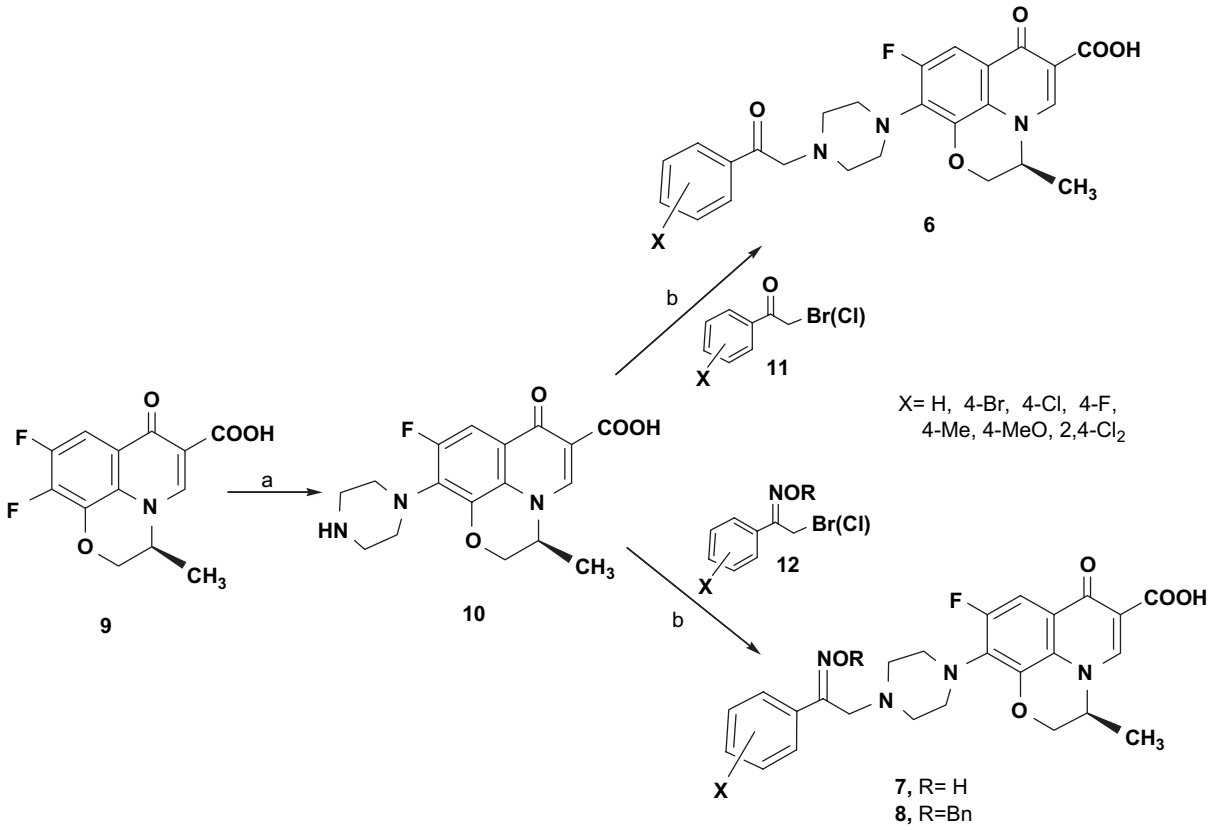
2. Chemistry

As summarized in Scheme 1, the *N*-substituted analogs of levofloxacin **6–8** were prepared by nucleophilic reaction of *N*-desmethyl levofloxacin **10** with phenacyl halides **11** or phenacyl halide oximes **12** employing reaction sequences previously described by us for the preparation of other *N*-substituted piperazinyl quinolones. Thus, phenacyl halide oxime derivatives **12** were synthesized by reaction of compound **11** with hydroxylamine hydrochloride or *O*-benzylhydroxylamine hydrochloride [10]. On the other hand, the piperazinyl quinolone (**10**, *N*-desmethyl levofloxacin) was prepared according to the known method [6], by the reaction of piperazine with (−)-9,10-difluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid **9**. Reaction of *N*-desmethyl levofloxacin **10** with phenacyl halides **11** or phenacyl halide oxime derivatives **12** in DMF, in the presence of $NaHCO_3$ at room temperature afforded corresponding ketones **6** and oxime derivatives **7** and **8**, respectively [10]. Structures and physicochemical data of compounds **6–8** are shown in Table 1.

3. Pharmacology

The newly synthesized compounds **6–8** were evaluated for their in vitro antibacterial activity against Gram-positive [*S. aureus* ATCC 25923, *S. aureus* ATCC 6538p, methicillin-resistant *S. aureus* (MRSA I and MRSA II, clinical isolates), *Staphylococcus epidermidis* ATCC 14940, *S. epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6051 and *Enterococcus feacalis* NCTC 6013] and Gram-negative bacteria [*Serratia marcescens* PTCC 1111, *Escherichia coli* ATCC 25922, *E. coli* NCTC 12900, *Klebsiella pneumoniae* ATCC 10031, *Salmonella typhi* ATCC 19430, *Shigella flexneri* NCTC 8516 and *Pseudomonas aeruginosa* ATCC 27853] using conventional agar-dilution method [16]. The minimum inhibitory concentration (MIC) values were determined by comparison to levofloxacin **4** and *N*-desmethyl levofloxacin **10** as reference drugs (Table 2).

Previous studies in quinolone field demonstrated that, in addition to the antibacterial activity, specific members of this drug family could display high cytotoxic activity against mammalian cells. These cytotoxic quinolones represent a potentially important source of new anticancer agents [17]. Therefore, the in vitro cytotoxic activity of selected



Scheme 1. Synthesis of compounds **6**–**8**. Reagents and conditions: (a) piperazine, pyridine, 100 °C, 18 h; (b) DMF, $NaHCO_3$, rt.

compounds **6b**, **6c**, **7b**, **7c** and **8a** was investigated in comparison with etoposide (ET) and cisplatin (CP) against breast carcinoma (MCF-7) cell line using MTT colorimetric assay [18]. MTT assay is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells. The relative cell viability was expressed as the mean percentage of viable cells compared with DMSO-treated cells and is presented in Fig. 2.

4. Results and discussion

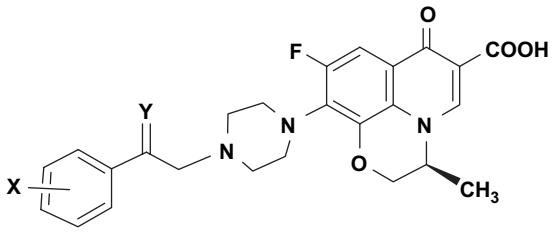
The minimum inhibitory concentrations (MICs, μg/mL) obtained for compounds **6**–**8** are presented in Table 2.

In general, Table 2 reveals that higher susceptibilities (lower MICs) were observed with Gram-positive and poorer susceptibilities with Gram-negative bacteria.

The MIC values of ketones **6** and oximes **7** against Gram-positive strains indicate that most compounds possessed a comparable or better activity (MIC = 0.06–1 μg/mL) with respect to *N*-desmethyl levofloxacin **10** and levofloxacin **4** (MIC = 0.25–4 μg/mL). It is noticeable that all ketones **6** and oximes **7** were exceptionally potent (MICs ≤ 1 μg/mL) against methicillin-resistant *S. aureus* (MRSA I and MRSA II) and their activities were comparable to those of parent quinolones levofloxacin **4** and *N*-desmethyl levofloxacin **10**. As is evident from the data, oximes **7b** and **7c** followed by ketones **6c** and **6e** are superior in inhibiting the growth of Gram-positives (MIC = 0.06–0.5 μg/mL), and their activities were found to be 4–8 times better than *N*-desmethyl levofloxacin **10** and equal or slightly better than levofloxacin **4**. In general, most compounds showed respectable activity (MIC = 0.12–4 μg/mL) against Gram-negative bacteria, with the exception for antibacterial activity against *K. pneumoniae*, *P. aeruginosa* and *S. marcescens*. In fact, all compounds including *N*-desmethyl levofloxacin **10** did not show significant activity against *P. aeruginosa* at concentrations ≤4 μg/mL. Compound **6a** was the most potent against *E. coli*, *K. pneumoniae*, *S. typhi* and *S. flexneri*. However, its activity was less than reference drugs against Gram-negative organisms.

In the terms of structure–activity relationship, the antibacterial activity profile against Gram-positive and Gram-negative bacteria was modulated by the phenethyl attachment at the 4-position of the piperazine ring in levofloxacin molecule. Bulky phenethyl group with different substituents are well tolerated in the term of Gram-positive activity. Thus, it can be proposed that for Gram-positive organisms, increasing molecular mass and bulkiness of substituent at C-10 position of levofloxacin molecule is not a barrier for interaction with target enzymes or for penetration into these bacteria. The overall activity profile of compounds **6a**–**g** and **7a**–**g** against Gram-positives demonstrated that there is a small difference in their MIC values and alteration of substitutions cannot markedly improve the activity against Gram-positive bacteria. In addition, in the term of Gram-negatives, better results were obtained with unsubstituted phenacyl derivative **6a**, and introduction of different substituent at *para*-position diminished anti-Gram-negative activity in most cases. It seems that this diminution of

Table 1
Structures and physicochemical data of levofloxacin analogs **6–8**



Compound	X	Y	Mp (°C)	Yield (%)	Formula (MW)	Elemental analysis Found (Calcd) %		
						C	H	N
6a	H	O	157–158	35	$C_{25}H_{24}FN_3O_5$ (465.47)	64.49 (64.51)	5.30 (5.20)	8.98 (9.03)
6b	4-Br	O	164–165	43	$C_{25}H_{23}BrFN_3O_5$ (544.37)	55.27 (55.16)	4.49 (4.26)	7.39 (7.72)
6c	4-Cl	O	160–162	47	$C_{25}H_{23}ClFN_3O_5$ (499.92)	59.86 (60.06)	4.28 (4.64)	8.78 (8.41)
6d	4-F	O	159–161	27	$C_{25}H_{23}F_2N_3O_5$ (483.46)	62.32 (62.11)	4.93 (4.80)	8.33 (8.69)
6e	4-CH_3O	O	129–131	29	$C_{26}H_{26}FN_3O_6$ (495.5)	63.34 (63.02)	5.18 (5.29)	8.65 (8.48)
6f	4-CH_3	O	165–167	33	$C_{26}H_{26}FN_3O_5$ (479.5)	65.37 (65.13)	5.63 (5.47)	8.51 (8.76)
6g	2,4-Cl_2	O	142–144	46	$C_{25}H_{22}Cl_2FN_3O_5$ (534.36)	56.38 (56.19)	4.26 (4.15)	8.01 (7.86)
7a	H	NOH	204–207	54	$C_{25}H_{25}FN_4O_5$ (480.49)	62.32 (62.49)	5.16 (5.24)	11.85 (11.66)
7b	4-Br	NOH	163–164	55	$C_{25}H_{24}BrFN_4O_5$ (559.38)	53.81 (53.68)	4.11 (4.32)	10.37 (10.02)
7c	4-Cl	NOH	175–177	51	$C_{25}H_{24}ClFN_4O_5$ (514.93)	58.22 (58.31)	4.52 (4.70)	11.06 (10.88)
7d	4-F	NOH	158–160	47	$C_{25}H_{24}F_2N_4O_5$ (498.48)	60.36 (60.24)	4.61 (4.85)	11.37 (11.24)
7e	4-CH_3O	NOH	148–150	49	$C_{26}H_{27}FN_4O_6$ (510.51)	61.09 (61.17)	5.46 (5.33)	10.99 (10.97)
7f	4-CH_3	NOH	169–171	39	$C_{26}H_{27}FN_4O_5$ (494.51)	62.97 (63.15)	5.41 (5.50)	11.47 (11.33)
7g	2,4-Cl_2	NOH	147–148	61	$C_{25}H_{23}Cl_2FN_4O_5$ (549.38)	54.76 (54.66)	4.29 (4.22)	10.52 (10.20)
8a	H	NOBn	163–165	65	$C_{32}H_{31}FN_4O_5$ (570.61)	67.48 (67.36)	5.22 (5.48)	9.65 (9.82)

activity is due to the steric or electronic effect of substituents at *para*-position. Comparison between MIC values of ketones **6a–g** and oximes **7a–g** revealed that oximation of ethanone linker caused a diminution in antibacterial activity against Gram-negative species, while maintaining the anti-Gram-positive activity. In addition, *O*-benzyloxime derivative **8a** did not show inhibitory activity at concentrations $\leq$4 μg/mL against all tested strains.

The quinolones are a well-known class of bactericidal agents that inhibit essential type II bacterial topoisomerases

Table 2
In vitro antibacterial activities of compounds **6–8** against selected strains[a] (MICs in μg/mL)

Compound	Gram-positive organisms								Gram-negative organisms						
	S.a. (I)	*S.a.* (II)	MRSA (I)	MRSA (II)	*S.e.* (I)	*S.e.* (II)	*B.s.*	*E.f.*	*S.m.*	*E.c.* (I)	*E.c.* (II)	*K.p.*	*S.t.*	*S.f.*	*P.a.*
6a	0.5	0.5	0.5	1	0.5	0.5	0.5	4	4	0.12	0.25	0.5	0.12	0.25	>4
6b	0.5	1	1	0.5	0.25	0.25	0.25	0.12	4	0.5	0.5	>4	0.5	0.5	>4
6c	0.5	0.5	0.25	0.25	0.12	0.25	0.12	0.06	4	0.5	1	>4	0.25	0.25	>4
6d	1	1	0.5	1	0.5	0.5	0.5	0.25	>4	1	2	>4	1	1	>4
6e	0.5	0.5	0.5	1	0.25	0.25	0.25	0.06	4	0.5	0.5	4	0.5	0.5	>4
6f	0.5	1	1	1	0.5	0.5	0.5	0.25	>4	1	0.5	>4	1	0.5	>4
6g	0.5	1	0.5	1	0.5	1	1	0.5	>4	1	2	>4	2	1	>4
7a	0.5	0.5	0.5	1	0.5	0.5	0.25	4	>4	4	4	4	0.5	0.5	>4
7b	0.5	0.5	0.5	0.5	0.12	0.25	0.12	0.06	>4	4	1	>4	0.5	0.5	>4
7c	0.5	0.25	0.5	0.25	0.12	0.25	0.25	0.06	>4	2	1	>4	0.5	0.5	>4
7d	1	1	1	1	0.5	0.5	0.25	0.25	>4	4	2	>4	1	1	>4
7e	1	0.5	1	0.5	0.5	0.5	0.12	0.06	>4	0.5	0.5	>4	0.5	0.5	>4
7f	1	0.5	0.5	0.5	0.5	0.5	0.5	0.25	>4	1	1	>4	1	1	>4
7g	0.5	0.5	0.5	0.5	0.5	0.5	1	4	>4	4	4	>4	4	4	>4
8a	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
4[b]	0.5	0.25	0.25	0.25	0.5	0.25	0.5	1	0.5	0.03	0.03	0.25	0.03	0.03	4
10[c]	4	2	2	2	1	1	1	0.06	1	0.12	0.12	0.25	0.06	0.06	>4

[a] *S.a.* (I): *Staphylococcus aureus* ATCC 25923, *S.a.* (II): *Staphylococcus aureus* ATCC 6538p, MRSA: methicillin-resistant *S. aureus* (clinical isolates I and II), *S.e.* (I): *Staphylococcus epidermidis* ATCC 14940, *S.e.* (II): *Staphylococcus epidermidis* ATCC 12228, *B.s.*: *Bacillus subtilis* ATCC 6051, *E.f.*: *Enterococcus feacalis* NCTC 6013, *S.m.*: *Serratia marcescens* PTCC 1111, *E.c.* (I): *Escherichia coli* ATCC 25922, *E.c.* (II): *Escherichia coli* NCTC 12900, *K.p.*: *Klebsiella pneumoniae* ATCC 10031, *S.t.*: *Salmonella typhi* ATCC 19430, *S.f.*: *Shigella flexneri* NCTC 8516, *P.a.*: *Pseudomonas aeruginosa* ATCC 27853.
[b] Levofloxacin.
[c] *N*-Desmethyl levofloxacin.

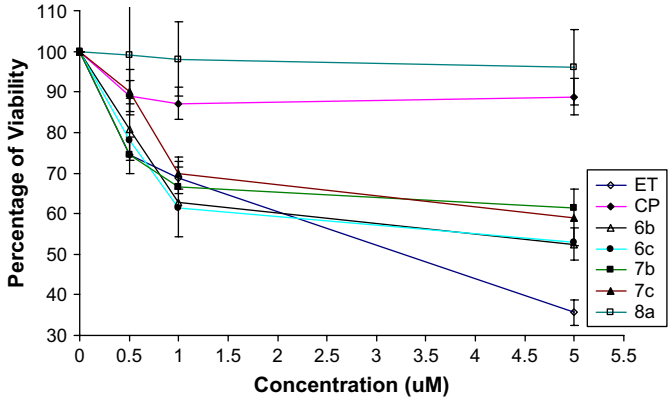


Fig. 2. Plot of concentration of selected compounds (**6b**, **6c**, **7b**, **7c** and **8a**) and reference drugs [etoposide (ET) and cisplatin (CP)] versus the cell viability of breast carcinoma (MCF-7).

such as DNA gyrase and topoisomerase IV [17,19]. Due to the structural and functional similarities between prokaryotic type II topoisomerases (DNA gyrase and topoisomerase IV) and the eukaryotic type II topoisomerase, the cytotoxicities of a selected series of these levofloxacin analogs were also evaluated. Thus, compounds that exhibited the most potent antibacterial activity against Gram-positives namely, **7b** and **7c**, and their corresponding ketones **6b** and **6c,** along with inactive compound **8a** were evaluated for in vitro cytotoxic activity against breast carcinoma (MCF-7) cell line in comparison with etoposide (ET) and cisplatin (CP). Fig. 2 shows the survival curve of MCF-7 cells after 48 h exposure to the different concentrations of test compounds (**6b**, **6c**, **7b**, **7c** and **8a**), etoposide (ET) and cisplatin (CP). For a better presentation of result, only the range of 0–5 μM is shown in this figure. As is shown, most potent antibacterial compounds **6b**, **6c**, **7b**, **7c** showed cytotoxic IC_{50}s of about 5.2 μM and statistically no difference with each other. However, etoposide kills 50% of these cells at 3.2 ± 0.3 μM and cisplatin killed about 70% of cells at the highest concentration. Compound **8a** (the inactive compound in antibacterial test) does not show any cytotoxicity on breast cell line and has not reached IC_{50} in the studied range of concentrations.

In conclusion, we have described synthesis and antibacterial evaluation of levofloxacin analogs carrying a 2-aryl-2-oxoethyl or a 2-aryl-2-oxyiminoethyl moiety attached to the piperazine ring at C-10 position. Biological data indicated that most compounds demonstrated comparable or better activity against Gram-positive bacteria than their parent quinolones, levofloxacin and *N*-desmethyl levofloxacin. Moreover, it can be concluded that for activity against Gram-positive organisms, increasing molecular mass and bulkiness of substituent at C-10 position of levofloxacin molecule is permitted and bulky phenethyl group with different substituents on C-10 piperazine ring, are well tolerated in the term of Gram-positive activity. Of the limited number of levofloxacin derivatives that we investigated for cytotoxic activity against breast carcinoma (MCF-7) cell line, the best antibacterial compounds, showed high cytotoxic activity and low selectivity as antibacterial agents. We anticipate that further modification of the substituents at C-10 piperazine ring and the quinolone nucleus, will, in general, regulate the selectivity of these compounds, potentially for antibacterial or cytotoxic activities. Such studies are now underway in our laboratories.

5. Experimental

5.1. Chemistry

Chemicals and all solvents used in this study were purchased from Merck AG and Aldrich Chemical. The phenacyl halide oxime derivatives **12** [10] and *N*-desmethyl levofloxacin **10** [6] were prepared according to the literature. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks). ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker 500 spectrometer, and chemical shifts are expressed as δ (ppm) with tetramethylsilane as internal standard. The electron impact mass spectra (EIMS) were run on a Finigan TSQ-70 spectrometer (Finigan, USA). The ionization energy was varied over a range of 20–70 eV. Elemental analyses were carried out on a HERAEUS CHN–O rapid elemental analyzer (GmbH, Germany) for C, H and N, and the results are within ±0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC. Yields are of purified product and were not optimized.

5.2. General procedure for the synthesis of compounds *6a–g*

A mixture of phenacyl halide **11** (0.55 mmol), *N*-desmethyl levofloxacin **10** (174 mg, 0.5 mmol) and $NaHCO_3$ (42 mg, 0.5 mmol) in DMF (5 mL), was stirred at room temperature for 6–10 h. After consumption of *N*-desmethyl levofloxacin **10** (monitored by TLC), water (20 mL) was added and the precipitate was filtered, washed with water and crystallized from methanol–chloroform (9:1) to give compound **6a–g**.

5.2.1. *10-[4-(2-Phenyl-2-oxoethyl)piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6a**)*

^{1}H NMR ($CDCl_3$) δ 14.82 (s, 1H, COOH), 8.66 (s, 1H, C_5–H), 8.05–8.00 (m, 2H, phenyl), 7.81 (d, 1H, C_8–H, $J = 12$ Hz), 7.75–7.70 (m, 1H, phenyl), 7.62–7.55 (m, 2H, phenyl), 4.60–4.35 (m, 3H, OCH_2CH), 3.98 (s, 2H, $COCH_2$), 3.58–3.30 (m, 8H, piperazine), 1.66 (d, 3H, CH_3, $J = 6.5$ Hz). IR (KBr, cm^{-1}): 3580–3300 (OH), 1722, 1672, 1622 (C=O). MS *m/z*: 465 (M^+).

5.2.2. *10-[4-[2-(4-Bromophenyl)-2-oxoethyl]piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6b**)*

^{1}H NMR ($CDCl_3$) δ 14.91 (s, 1H, COOH), 8.64 (s, 1H, C_5–H), 7.75 (d, 1H, C_8–H, $J = 12.1$ Hz), 7.86 (d, 2H, phenyl, $J = 8.0$ Hz), 7.69 (d, 2H, phenyl, $J = 8.0$ Hz), 4.6–4.34 (m, 3H, OCH_2–CH), 3.93 (s, 2H, $COCH_2$), 3.58–3.35 (m, 8H,

piperazine), 1.62 (d, 3H, CH_3, $J = 6.4$ Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.76 (CH_3), 50.94 (d, C_2' and C_6' piperazine, $^4J_{C-F} = 4.06$ Hz), 54.42 (C_3' and C_5' piperazine), 55.96 (C_3), 65.12 ($-CH_2$), 68.58 (C_2), 105.72 (d, C_8, $^2J_{C-F} = 24.13$ Hz), 108.26 (C_6), 120.94 (d, C_{13}, $^3J_{C-F} = 9.12$ Hz), 125.14 (C_{12}), 129.03 (C_4'' phenyl), 130.25 (C_3'' and C_5'' phenyl), 132.06 (d, C_{10}, $^2J_{C-F} = 14.01$ Hz), 132.36 (C_2'' and C_6'' phenyl), 133.36, 135.03 (C_1'' phenyl), 141.03 (d, C_{11}, $^3J_{C-F} = 6.75$ Hz), 145.07 (C_5), 156.02 (d, C_9, $^1J_{C-F} = 253.25$ Hz), 167.59 (COOH), 177.55 (C_7), 195.93 (C=O, phenacyl). IR (KBr, cm^{-1}): 3580–3300 (OH), 1725, 1695, 1619 (C=O). MS *m/z*: 543 and 545 (M^+).

*5.2.3. 10-[4-[2-(4-Chlorophenyl)-2-oxoethyl]piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6c**)*

^1H NMR ($CDCl_3$) δ 14.91 (s, 1H, COOH), 8.63 (s, 1H, C_5–H), 7.95 (d, 2H, phenyl, $J = 8.4$ Hz), 7.77 (d, 1H, C_8–H, $J = 12$ Hz), 7.52 (d, 2H, phenyl, $J = 8.4$ Hz), 4.58–4.35 (m, 3H, OCH_2CH), 3.93 (s, 2H, $COCH_2$), 3.58–3.30 (m, 8H, piperazine) and 1.62 (d, 3H, CH_3, $J = 6.4$ Hz). IR (KBr, cm^{-1}): 3600–3300 (OH), 1719, 1680, 1620 (C=O). MS *m/z*: 499 (M^+).

*5.2.4. 10-[4-[2-(4-Fluorophenyl)-2-oxoethyl]piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6d**)*

^1H NMR ($CDCl_3$) δ 14.91 (s, 1H, COOH), 8.63 (s, 1H, C_5–H), 7.76 (d, 1H, C_8–H, $J = 12$ Hz), 7.66–7.61 (m, 2H, phenyl), 7.18 (t, 2H, phenyl, $J = 8.7$ Hz), 4.56–4.33 (m, 3H, OCH_2CH), 3.95 (s, 2H, $COCH_2$), 3.60–3.35 (m, 8H, piperazine) and 1.63 (d, 3H, CH_3, $J = 6.4$ Hz). IR (KBr, cm^{-1}): 3590–3300 (OH), 1719, 1679, 1618 (C=O). MS *m/z*: 483 (M^+).

*5.2.5. 10-[4-[2-(4-Methoxyphenyl)-2-oxoethyl]piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6e**)*

^1H NMR ($CDCl_3$) δ 14.5 (s, 1H, COOH), 8.70 (s, 1H, C_5–H), 7.80 (d, 1H, C_8–H, $J = 12.3$ Hz), 8.09 (d, 2H, phenyl, $J = 8.7$ Hz), 6.98 (d, 2H, phenyl, $J = 8.7$ Hz), 4.6–4.4 (m, 3H, OCH_2CH), 3.92 (s, 5H, OCH_3 and $COCH_2$), 3.6–3.4 (m, 8H, piperazine), 1.65 (d, 3H, CH_3, $J = 6.2$ Hz). IR (KBr, cm^{-1}): 3580–3300 (OH), 1712, 1685, 1619 (C=O). MS *m/z*: 495 (M^+).

*5.2.6. 10-[4-[2-(4-Methylphenyl)-2-oxoethyl]piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6f**)*

^1H NMR ($CDCl_3$) δ 14.99 (s, 1H, COOH), 8.64 (s, 1H, C_5–H), 8.07 (d, 2H, phenyl, $J = 7.5$ Hz), 7.79 (d, 1H, C_8–H, $J = 12.2$ Hz), 7.64 (d, 2H, phenyl, $J = 7.5$ Hz), 4.6–4.3 (m, 3H, OCH_2–CH), 3.94 (s, 2H, $COCH_2$), 3.60–3.4 (m, 4H, piperazine), 2.81 (m, 4H, piperazine), 2.38 (s, 3H, $CH_3C_6H_4$), 1.66 (d, 3H, CH_3, $J = 6.1$ Hz). IR (KBr, cm^{-1}): 3580–3300 (OH), 1722, 1666, 1621 (C=O). MS *m/z*: 479 (M^+).

*5.2.7. 10-[4-[2-(2,4-Dichlorophenyl)-2-oxoethyl]piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**6g**)*

^1H NMR ($CDCl_3$) δ 14.8 (s, 1H, COOH), 8.68 (s, 1H, C_5–H), 7.91 (d, 1H, phenyl, $J = 8.4$ Hz), 7.78 (d, 1H, C_8–H, $J = 11.8$ Hz), 7.55–7.42 (m, 2H, phenyl), 4.65–4.40 (m, 3H, OCH_2–CH), 3.91 (s, 2H, $COCH_2$), 3.75–3.65 (m, 4H, piperazine), 3.57–3.40 (m, 4H, piperazine), 1.67 (d, 3H, CH_3, $J = 6$ Hz). IR (KBr, cm^{-1}): 3580–3300 (OH), 1716, 1661, 1618 (C=O). MS *m/z*: 533 (M^+).

*5.3. General procedure for the synthesis of compounds **7a–g***

A mixture of compound **12** (0.33 mmol), *N*-desmethyl levofloxacin **10** (104 mg, 0.3 mmol) and $NaHCO_3$ (26 mg, 0.3 mmol) in DMF (3 mL), was stirred at room temperature for 6–10 h. After consumption of *N*-desmethyl levofloxacin **10** (monitored by TLC), water (20 mL) was added and the precipitate was filtered, washed with water and crystallized from ethanol–chloroform (9:1) to give compound **7a–g**.

*5.3.1. 10-[4-(2-Phenyl-2-hydroxyiminoethyl)piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-[1,4]benzoxazine-6-carboxylic acid (**7a**)*

^1H NMR ($CDCl_3$) δ 15.1 (s, 1H, COOH), 10.94 (s, 1H, NOH), 8.96 (s, 1H, C_5–H), 7.57 (d, 1H, C_8–H, $J = 12.2$ Hz), 7.65–7.62 (m, 2H, phenyl), 7.44–7.33 (m, 3H, phenyl), 4.90 (br s, 2H, CH_2), 4.58–4.3 (m, 3H, OCH_2CH), 3.45–3.20 (m, 8H, piperazine), 1.44 (d, 3H, CH_3, $J = 6$ Hz). IR (KBr, cm^{-1}): 3580–3300 (OH), 1718, 1619 (C=O). MS *m/z*: 480 (M^+).

*5.3.2. 10-[4-(2-(4-Bromophenyl)-2-hydroxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**7b**)*

^1H NMR ($CDCl_3$) δ 8.62 (s, 1H, C_5–H), 7.73 (d, 1H, C_8–H, $J = 12.4$ Hz), 7.60–7.45 (m, 4H, phenyl), 4.55–4.30 (m, 3H, OCH_2–CH), 3.91 (s, 2H, CNOH–CH_2), 3.40–3.24 (m, 4H, piperazine), 2.95–2.64 (m, 4H, piperazine), 1.61 (d, 3H, CH_3, $J = 6$ Hz). IR (KBr, cm^{-1}): 3625–3250 (OH), 1724, 1623 (C=O). MS *m/z*: 558 and 560 (M^+).

*5.3.3. 10-[4-(2-(4-Chlorophenyl)-2-hydroxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**7c**)*

^1H NMR ($CDCl_3$) δ 15.1 (s, 1H, COOH), 10.7 (s, 1H, NOH), 8.71 (s, 1H, C_5–H), 7.68 (d, 1H, C_8–H, $J = 12$ Hz), 7.44–7.35 (m, 4H, phenyl), 4.65–4.30 (m, 3H, OCH_2CH), 3.6–3.3 (m, 6H, 4H piperazine and 2H, CNOH–CH_2), 2.9–2.6 (m, 4H, piperazine), 1.59 (d, 3H, CH_3, $J = 6$ Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ 18.77 (CH_3), 51.03 (C_2' and C_6' piperazine), 54.03 (C_3' and C_5' piperazine), 55.67 ($-CH_2$), 62.30 (C_3), 68.89 (C_2), 104.10 (d, C_8, $^2J_{C-F} = 23.75$ Hz), 107.52 (C_6), 120.59 (d, C_{13}, $^3J_{C-F} = 9.13$ Hz), 125.67 (C_{12}), 128.69 (C_3'' and C_5'' phenyl), 128.94, 131.30 (C_2'' and C_6'' phenyl), 132.40 (d, C_{10}, $^2J_{C-F} = 13.62$ Hz), 132.95 (C_1'' phenyl),

133.76 (C_4'' phenyl), 141.14 (d, C_{11}, $^3J_{C-F} = 6.87$ Hz), 147.05 (C_5), 151.97 (C=N), 156.33 (d, C_9, $^1J_{C-F} = 245.37$ Hz), 166.92 (COOH), 177.25 (C_7). IR (KBr, cm^{-1}): 3580–3300 (OH), 1716, 1618 (C=O). MS *m/z*: 514 (M^+).

*5.3.4. 10-[4-(2-(4-Fluorophenyl)-2-hydroxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**7d**)*

^{1}H NMR ($CDCl_3$) δ 14.97 (s, 1H, COOH), 10.85 (s, 1H, NOH), 8.64 (s, 1H, C_5–H), 7.78 (d, 1H, C_8–H, $J = 12.2$ Hz), 7.76–7.70 (m, 2H, phenyl), 7.20–7.10 (m, 2H, phenyl), 4.55–4.30 (m, 3H, OCH_2–CH), 3.45 (s, 2H, CNOH–CH_2), 3.44–3.30 (m, 4H, piperazine), 2.70 (br s, 4H, piperazine), 1.65 (d, 3H, CH_3, $J = 6$ Hz). IR (KBr, cm^{-1}): 3600–3250 (OH), 1713, 1619 (C=O). MS *m/z*: 498 (M^+).

*5.3.5. 10-[4-(2-(4-Methoxyphenyl)-2-hydroxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**7e**)*

^{1}H NMR ($CDCl_3$) δ 14.91 (s, 1H, COOH), 10.91 (s, 1H, NOH), 8.95 (s, 1H, C_5–H), 7.56 (d, 1H, C_8–H, $J = 12.2$ Hz), 7.71 (d, 2H, phenyl, $J = 8.8$ Hz), 6.96 (d, 2H, phenyl, $J = 8.8$ Hz), 4.95–4.85 (m, 1H, C_3–H), 4.58–4.33 (m, 2H, OCH_2), 3.79 (s, 3H, OCH_3), 3.38 (s, 2H, CNOH–CH_2), 3.36–3.20 (m, 8H, piperazine), 1.44 (d, 3H, CH_3, $J = 6.3$ Hz). IR (KBr, cm^{-1}): 3580–3300 (OH), 1712, 1620 (C=O). MS *m/z*: 510 (M^+).

*5.3.6. 10-[4-(2-(4-Methylphenyl)-2-hydroxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**7f**)*

^{1}H NMR ($CDCl_3$) δ 14.91 (s, 1H, COOH), 8.61 (s, 1H, C_5–H), 7.70 (d, 1H, C_8–H, $J = 12.2$ Hz), 7.4–7.2 (m, 4H, phenyl), 4.54–4.25 (m, 3H, OCH_2–CH), 3.68 (br s, 2H, NOH–CH_2), 3.4–3.2 (m, 8H, piperazine), 2.35 (s, 3H, $CH_3C_6H_4$), 1.58 (d, 3H, CH_3, $J = 6$ Hz). ^{13}C NMR (125 MHz, DMSO-d_6) δ 18.76 (3-CH_3), 21.71 (4-CH_3–phenyl), 51.03 (C_2' and C_6' piperazine), 54.07 (C_3' and C_5' piperazine), 55.67 (–CH_2), 62.53 (C_3), 68.89 (C_2), 104.11 (d, C_8, $^2J_{C-F} = 23.38$ Hz), 107.51 (C_6), 120.59 (d, C_{13}, $^3J_{C-F} = 9.62$ Hz), 125.66 (C_{12}), 129.15 (C_3'' and C_5'' phenyl), 129.31 (C_2'' and C_6'' phenyl), 131.37 (C_1'' phenyl), 132.87 (d, C_{10}, $^2J_{C-F} = 13.75$ Hz), 138.57 (C_4'' phenyl), 141.16 (d, C_{11}, $^3J_{C-F} = 7.00$ Hz), 147.05 (C_5), 152.82 (C=N), 156.34 (d, C_9, $^1J_{C-F} = 245.50$ Hz), 166.92 (COOH), 177.26 (C_7). IR (KBr, cm^{-1}): 3580–3300 (OH), 1712 (C=O). MS *m/z*: 494 (M^+).

*5.3.7. 10-[4-(2-(2,4-Dichlorophenyl)-2-hydroxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**7g**)*

^{1}H NMR ($CDCl_3$) δ 15.0 (s, 1H, COOH), 8.62 (s, 1H, C_5–H), 7.74 (d, 1H, C_8–H, $J = 12.4$ Hz), 7.48 (d, 1H, phenyl, $J = 2$ Hz), 7.39 (dd, 1H, phenyl, $J = 8$ and 2 Hz), 7.16 (d, 1H, phenyl, $J = 8$ Hz), 4.5–4.3 (m, 3H, O–CH_2–CH), 3.40 (s, 2H, CNOH–CH_2), 3.4–3.28 (m, 4H, piperazine), 2.6 (br s, 4H, piperazine), 1.61 (d, 3H, CH_3, $J = 6$ Hz). ^{13}C NMR (125 MHz, DMSO-d_6 + $CDCl_3$) δ 18.70 (CH_3), 50.89 (C_2' and C_6' piperazine), 54.07 (C_3' and C_5' piperazine), 55.71 (–CH_2), 61.69 (C_3), 68.48 (C_2), 105.21 (d, C_8, $^2J_{C-F} = 23.98$ Hz), 107.91 (C_6), 121.02 (d, C_{13}, $^3J_{C-F} = 9.29$ Hz), 126.67 (C_{12}), 127.09 (C_5'' phenyl), 129.45 (C_3'' phenyl), 130.73 (C_6'' phenyl), 132.87 (C_2'' phenyl), 132.97 (C_1'' phenyl), 134.70 (C_4'' phenyl), 139.95 (d, C_{11}, $^3J_{C-F} = 7.50$ Hz), 145.30 (C_5), 151.72 (C=N), 156.62 (d, C_9, $^1J_{C-F} = 246.25$ Hz), 167.41 (COOH), 177.40 (C_7). IR (KBr, cm^{-1}): 3580–3300 (OH), 1715, 1618 (C=O). MS *m/z*: 548 (M^+).

*5.4. 10-[4-(2-Phenyl-2-phenylmethoxyiminoethyl)-piperazin-1-yl]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid (**8a**)*

A mixture of 2-bromoacetophenone *O*-benzyloxime (100 mg, 0.33 mmol), *N*-desmethyl levofloxacin **10** (104 mg, 0.3 mmol) and $NaHCO_3$ (26 mg, 0.3 mmol) in DMF (4 mL) was stirred at room temperature overnight. After consumption of *N*-desmethyl levofloxacin **10** (monitored by TLC), water (20 mL) was added and extracted with $CHCl_3$. The organic layer was washed (H_2O), dried (Na_2SO_4) and evaporated in vacuo. The residue was crystallized from methanol–chloroform (9:1) to give compound **8a**. ^{1}H NMR ($CDCl_3$) δ 15.05 (s, 1H, COOH), 8.65 (s, 1H, C_5–H), 7.83 (m, 2H, phenyl), 7.68 (d, 1H, C_8–H, $J = 12$ Hz), 7.50–7.24 (m, 8H, phenyl), 5.26 (s, 2H, CNO–CH_2), 4.60–4.30 (m, 3H, OCH_2CH), 3.77 (br s, 2H, ONC–CH_2), 3.45–3.2 (m, 4H, piperazine), 2.75–2.55 (m, 4H, piperazine) and 1.59 (d, 3H, CH_3, $J = 6$ Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ 18.78 (CH_3), 51.15 (d, C_2' and C_6' piperazine, $^4J_{C-F} = 3.75$ Hz), 52.45 (–CH_2), 54.18 (C_3' and C_5' piperazine), 56.01 (C_3), 68.56 (C_2), 76.92 (OCH_2–Ph), 105.69 (d, C_8, $^2J_{C-F} = 24.63$ Hz), 108.22 (C_6), 120.63 (d, C_{13}, $^3J_{C-F} = 9.3$ Hz), 125.14 (C_{12}), 127.26 (C_2''' and C_6''' phenyl), 128.28 (C_4''' phenyl), 128.60 (C_3'' and C_5'' phenyl), 128.71 (C_3''' and C_5''' phenyl), 128.79 (C_2'' and C_6'' phenyl), 129.52 (C_4'' phenyl), 133.67 (d, C_{10}, $^2J_{C-F} = 14.13$ Hz), 136.05 (C_1'' phenyl), 138.20 (C_1''' phenyl), 139.57 (d, C_{11}, $^3J_{C-F} = 6.9$ Hz), 145.07 (C_5), 155.10 (C=N), 156.40 (d, C_9, $^1J_{C-F} = 248.12$ Hz), 167.62 (COOH), 177.56 (C_7). IR (KBr, cm^{-1}): 3580–3300 (OH), 1715, 1619 (C=O). MS *m/z*: 570 (M^+).

5.5. Antibacterial activity

Compounds **6–8** were evaluated for their antibacterial activity using conventional agar-dilution method [16]. Two-fold serial dilutions of the compounds and reference drugs were prepared in Muller–Hinton agar. Drugs (6.4 mg) were dissolved in dimethylsulfoxide (DMSO, 1 mL) and the solution was diluted with water (9 mL) without precipitation. Further progressive double dilution with melted Muler–Hinton agar was performed to obtain the required concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 μg/mL. Petri dishes were incubated with $1–5 \times 10^4$ colony forming units (cfu) and incubated at 37 °C for 18 h. The minimum inhibitory concentration (MIC) was the lowest concentration of the test compound, which resulted in no visible growth on

the plate. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO at the same dilutions as used in the experiment.

5.6. *In vitro* cytotoxicity assay

The MCF-7 human breast carcinoma cell line used in this experiment was obtained from the Iran Pasture Institute Cell Line Bank (Pasture Inst., Tehran, Iran). Cells were grown in the DMEM/F12 media supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. For the experiments, cells were plated at a density of 50,000 cells/mL in 96-well plates. One day after seeding, the cells were treated with the medium containing the compounds at 0, 0.5, 1, 5, 10, 40, 70 and 100 μM concentrations for 2 days, and finally the MTT reduction assay was carried out [18]. All compounds were dissolved in DMSO (final concentration 1%) and complete medium. Untreated cells were used as controls. Each experiment was carried out in quadruplicate.

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